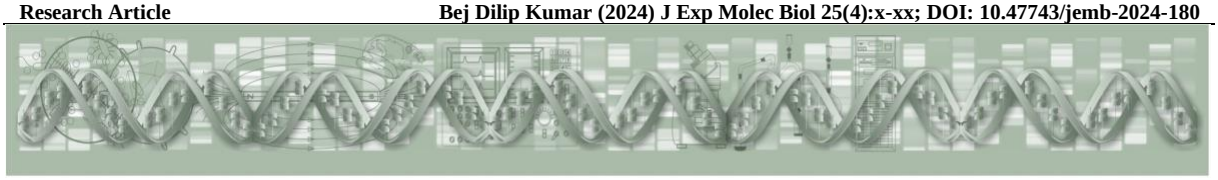

# MOLECULAR CLONING AND QUANTITATIVE mRNA EXPRESSION OF *sox9* GENE IN THE GONADAL DEVELOPMENTAL PERIOD OF THE FISH COBALTCAP SILVERSIDE *HYPOATHERINA TSURUGAE*

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## Abstract

*Sox9* is a transcription factor of high mobility group (HMG) box family DNA binding domain. It plays a crucial role in gonadogenesis during embryonic developmental period. 1454 bp of *sox9* mRNA transcript of *Hypoatherina tsurugae* (D. S. Jordan & Starks) was cloned and sequenced. It consists of an open reading frame (ORF) of 1436 bp, that encodes a 479 aa protein, found to be identical to the HMG box of other fish species. A phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fishes across various taxa available in the NCBI database and using as outgroup *Acipenser sinensis*. The tree shows a high homology of *sox9* from *H. tsurugae* with that from *Maelanotaenia boesemani*, the two forming a single clade. The expression of *sox9* was studied in *amhy*+ (male) individuals. It begins from baseline at 0 wah (week after hatching) and is expressed in an increasing fashion. In *amhy*- (female) individuals it is highly expressed at initial stage (0 wah) and the expression reaches its peak at 2 wah then declines, indicating the low expression needed for differentiation of the female sex organs. The histological sections of gonads were studied in different stages of biweekly collected larvae during the sex determination/differentiation period and it showed that differentiation of gonads male/female is decided at 6 wah. In this stage the primary oocytes are clearly recognized and it correlates with the expression of *sox9* genes. These finding add to the knowledge for a better understanding of molecular mechanisms of sex determination and differentiation period in fishes.

**Keywords**: Atheriniformes, *sox9*, *Hypoatherina tsurugae*, Gonadal development

## Introduction

It has been reported that the *amhy* gene (Y chromosome-linked anti-Müllerian hormone) has a critical role in male sex determination of an old world Silverside, *Hypoatherina tsurugae* (D. S. Jordan & Starks) (Bej *et al*. 2017). Many other genes/transcription factors have also been described as master sex determining genes in various fishes (Hattori *et al*. 2013, Yano *et al*. 2012, Takehana *et al*. 2014, Matsuda *et al*. 2002, Myosho *et al*. 2012). From these references, it is clear that the genetic machinery of fishes which control gonadal development is very diverse and is not limited to a particular gene/transcription factor as most interestingly reported for the *sdY*, an immune related gene that can crosstalk as sex determining gene in Salmonidae (Yano *et al*. 2012).

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Sox (Sry related high mobility group box) are a gene family of transcription factors that possess an important role in reproduction and development of gonads (Hu *et al*. 2021). *Sox9* is a member of Sox-family that serves a crucial role in testis formation besides other function like cartilage formation (Healy *et al*. 1999, Jakubiczka *et al*. 2010). This is a potential candidate gene in fish that may drive downstream development of gonads after being triggered by master sex determining gene during gonadal differentiation period. Sekido and Lovell-Badge (2008) believed this gene is to be the main effector of *sry* gene to regulate the downstream pathway. Studies show that the mutation in *sox9* gene results in abnormal bone formation and even sex reversal (Jakubiczka *et al*. 2010, Georg *et al*. 2010).

*Hypoatherina tsurugae*, commonly called cobaltcap silverside, has very little information about its reproductive biology and sex differentiation. In this species besides the *amhy* gene (Bej *et al*. 2017), the expression of other genes has not been studied during the gonadal determination/differentiation period. So, the objective of this paper is to study the expression pattern of *sox9* gene in this species and its role during the gonadal development period.

## Materials And Methods

About 100 matured wild cobaltcap silversides were collected using a hand net and were subsequently reared in a 500 liter tank to obtain gametes and offsprings for experiments. The tanks were supplied with filtered natural sea water at a rate of 100 ml/min. Larvae were fed rotifers *Branchionus rotundiformis* and *Artemia* nauplii from the first day to satiation twice daily and gradually weaned into powdered marine fish food (AQUEON, Franklin, WI).

Genomic DNA was isolated from caudal fin tissue following a protocol described by Aljanabi and Martinez (1997). The genotyping of larvae to know their sex (male / female) were performed using primers Amh 613 F and Amh 35 R (Bej *et al*. 2017).

### Cloning of *sox9* gene

For cloning, total RNA was isolated from *amhy*+ individuals testis using TRIzol (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instruction. 1 µg of total RNA per sample was reverse transcribed using SuperScript III (Thermo Fisher Scientific) with Oligo-(dT) primers (Merk Millipore, Darmstadt, German) in 20 µl reactions. The PCR was performed according to the following conditions: 3 min at 94 ºC, 30 cycles of 30 sec at 94 ºC, 45 sec at 60 ºC and 2.5 min at 72 ºC, then followed by a final elongation for 5 min at 72 ºC. PCR products were electrophoresed in 1% agarose gel, purified, and sequenced in an ABI PRISM 3100 capillary sequencer (Life Technologies, Carlsbad, CA) using the BigDye Terminator method. Sequences were analyzed with GENETYX version 11.0 (GENETYX, Tokyo, Japan). All primers are listed in Table 1.

Table 1. List of Primers used in cloning and qRT-PCR (designed for this study)

| Sl.No | Name of Primers | Sequences |
|---|---|---|
| **1** | Sox1 F | 5'-TTCGCATGAATCTCCTCGACC-3' |
| **2** | Sox last R | 5'-TCCTCAGGGCCTGGACACAG -3' |
| **3** | *sox* RT 809 F | 5'-GGTGAGCTGAGCA GCGAGGT-3' |

| | | |
|---|---|---|
| **4** | *sox* RT 935 R | 5'-TGCAGGTTGAAGGAGCCGTA-3' |
| **5** | *β-actin*Fw17 | 5'- GCCTGAAACCGGTTCCCTT-3' |
| **6** | *β-actin*Rv1838 | 5'-TTTTCGGAACACATGTGCACT-3' |
| **7** | *β-actin* RT F | 5'-GTGCTGTCTTCCCCTCCATC-3' |
| **8** | *β-actin* RT R | 5'-TCTTGCTCTGGGCTTCATCA-3' |



## Real-Time/Quantitative PCR (qRT-PCR)

For expression studies, total RNA was isolated from *amhy*+ and *amhy*- individuals every two weeks after hatch (wah), namely 0 wah, 2 wah, 4 wah, 6 wah, 8 wah and 10 wah. The expression level of mRNA transcripts was analyzed by qRT-PCR using specific RT primers designed for *sox9* locus using conditions from a previous study (Bej *et al*. 2017). The β-*actin* gene was taken as an endogenous control because of its stability during sex determination/differentiation period, using the primers from (Chapman *et al*. 2015) (Tab. 1).

## Sequence analysis

The multiple alignment software Clustal W was used for the alignment of nucleotide sequences and their deduced amino acid sequences. The phylogenetic tree was constructed using MEGAX with Maximum Likelihood, the initial tree inferred with the Neighbour-Joining and the BioNJ algorithms, and the Tamura-Nei model. The model was determined also using MEGAX. The Confidence in the tree topology was assessed with 1,000 bootstrap replicates.

## Statistical analysis

In qRT-PCR expression studies, per each time point five to seven samples were taken. The differences in gene expression between groups were analyzed by ANOVA followed by a Tukey test using GraphPad prism (v.6.0; GraphPad software, San Diego, CA). Differences in gene expression were considered as statistically significant at $p < 0.05$.

## Histological analysis of gonadal sex differentiation

Trunk samples were dehydrated through an ascending ethanol series (70%, 90%, 99%, and 100%), cleared in xylene, embedded in Paraplast Plus (McCormick Scientific, St. Louis, MO), sectioned serially with a thickness of 5 µm, and stained with hematoxylin and eosin. Stages of gonadal sex differentiation were determined by light microscopy using histological criteria for another atheriniform, the pejerrey *Odontesthes bonariensis* (Ito *et al*. 2005).

## Data Accessibility

DNA sequences: GenBank accessions; *Hypoatherina tsurugae sox*9 [PP108745], *Acanthochromis polyacanthus sox9* [XM_022211835.2], *Amphiprion ocellaris sox9* [XM_023286694.3], *Anabas testudineus sox9* [XM_026358727.1], *Anarrhichthys ocellatus sox9* [XM_031867589.1], *Anoplopoma fimbria sox9* [XM_054606768.1], *Astatotilapia calliptera sox9* [XM_026164918.1], *Chelmon rostratus sox9* [XM_041957848.1], *Cololabis*

*saira sox9* [XM_061712632.1], *Cottoperca gobio sox9* [XM_029437533.1], *Dicentrarchus labrax sox9* [XM_051389381.1], *Epinephelus fuscoguttatus sox9* [XM_049561363.1], *Etheostoma cragini sox9* [XM_034894690.1], *Etheostoma spectabile sox9* [XM_032544694.1], *Haplochromis burtoni sox9* [XM_005936187.2], *Hippoglossus hippoglossus sox9* [XM_034593479.1], *Kryptolebias marmoratus sox9* [ XM_017425448.3], *Larimichthys crocea sox9* [MH996432.1], *Lates calcarifer sox9* [KR492508.1], *Mastacembelus armatus sox9* [XM_026325302.2], *Maylandia zebra sox9* [XM_004559402.2], *Melanotaenia boesemani sox9* [XM_041970542.1], *Micropterus salmoides sox9* [XM_038701099.1], *Morone saxatilis sox9* [XM_035655588.1], *Nematolebias whitei sox9* [XM_037689002.1], *Neolamprologus brichardi sox9* [XM_006791412.2], *Odontesthes bonariensis sox9* [AY319415.4], *Oncorhynchus mykiss sox9* [AB006448.1], *Oreochromis niloticus sox9* [XM_003450119.4], *Oryzias latipes sox9* [NM_001105086.1], *Oryzias melastigma sox9* [XM_024272555.2], *Paralichthys olivaceus sox9* [KY924902.1], *Perca fluviatilis sox9* [XM_039825773.1], *Plectropomus leopardus sox9* [XM_042504339.1], *Poecilia formosa sox9* [XM_007556363.2], *Pseudoliparis swirei sox9* [XM_056407289.1], *Pundamilia nyererei sox9* [XM_005744343.1], *Sander lucioperca sox 9* [XM_031312361.2], *Scatophagus argus sox9* [XM_046415919.1], *Seriola dumerili sox9* [XM_022752333.1], *Simochromis diagramma sox9* [XM_040049522.1], *Siniperca chuatsi sox9* [XM_044181768.1], *Solea senegalensis sox9* [XM_044024558.1], *Stegastes partitus sox9* [XM_008303357.1], *Thunnus albacares sox9* [XM_044332285.1], *Thunnus maccoyii sox9* [XM_042393607.1], *Toxotes jaculatrix sox9* [XM_041062045.1], *Trematomus bernacchii sox9* [XM_034143142.1], *Xiphias gladius sox9* [XM_040123617.1] and *Acipenser sinensis sox9* [KJ526295.1]

## Results

### Sequence analysis of *sox9*

The isolated *sox9* cDNA was 1454 bp with an open reading frame (ORF) of 1436 bp, encoding a 479 aa protein (GenBank Accession number – PP108745) (Fig. 1). It shows a close similarity at nucleotide level to the HMG box of *sox9* gene of *Melanotaenia boesemani* (96.42%), *Stegastes partitus* (92.43%), *Odontesthes bonariensis* (92.42%), *Xiphias gladius* (91.01%), *Seriola dumerili* (90.98%), *Lates calcarifer* (90.52%), *Dicentrarchus labrax* (90.51%) and *Oreochromis niloticus* (89.40%). By using the Clustal W software, the 479 amino acid sequence of *H. tsurugae* was aligned with nine other fish species. The results showed that the homology was high: *Melanotaenia boesemani* (98.54%), *Odontesthes bonariensis* (96.66%), *Lates calcarifer* (96.86%), *Xiphias gladius* (96.25%), *Dicentrarchus labrax* (95.82%), *Plectropomus leopardus* (95.41%), *Perca flavescens* (95.2%) and *Oreochromis niloticus* (92.99%) (Fig. 2).

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atgaatctcctcgacccatacctgaagatgacagaagaacaggagaagtgtcactctgac
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 A  P  S  P  S  M  S  E  D  S  A  G  S  P  C  P  S  G  S  G
tcggacactgaaaacacccggccgtccgacaaccacctcctcggaggtcctgactacaag
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 I  F  K  A  L  Q  Q  A  D  S  P  A  S  S  M  G  E  V  H  S
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agccagaggccgatgtacaccccgattgctgacagcaccggggtgccctctgtgccgcag
 S  Q  R  P  M  Y  T  P  I  A  D  S  T  G  V  P  S  V  P  Q
```

**Fig. 1.** *sox9* gene of *Hypoatherina tsurugae* with complete CDS

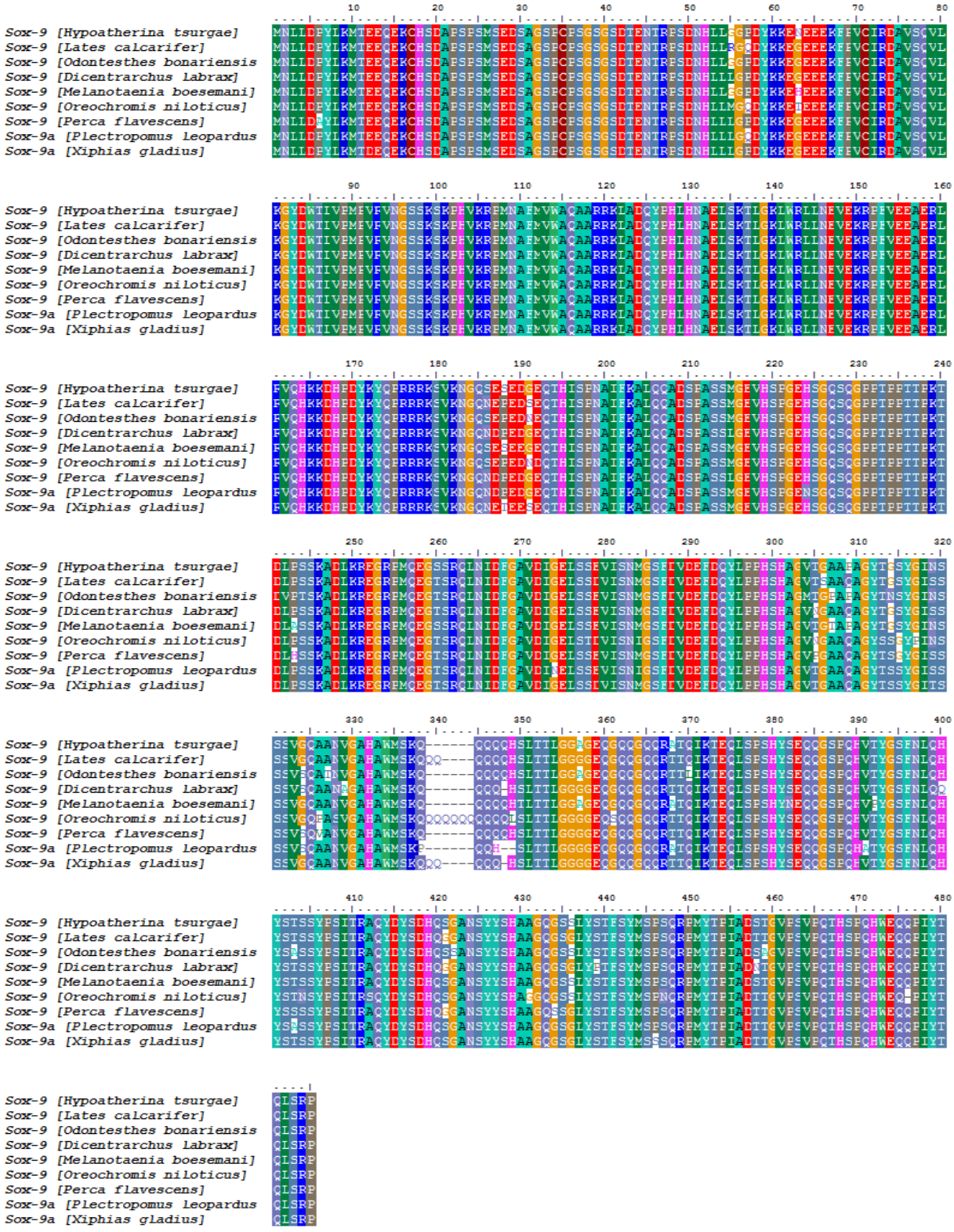


**Fig. 2**. Deduced amino acids sequence of *H. tsurugae sox9* gene aligned with other ortholog sequences

A phylogenetic tree was constructed by comparing the mRNA sequence of 50 different fish species across various taxa available in the NCBI database and taking *Acipenser sinensis* as outgroup (Fig. 3). The tree shows a high homology of *H. tsurugae sox9* with *Maelanotaenia boesemani sox9*, the two being sister taxa as they both belong to the order Atheriniformes.

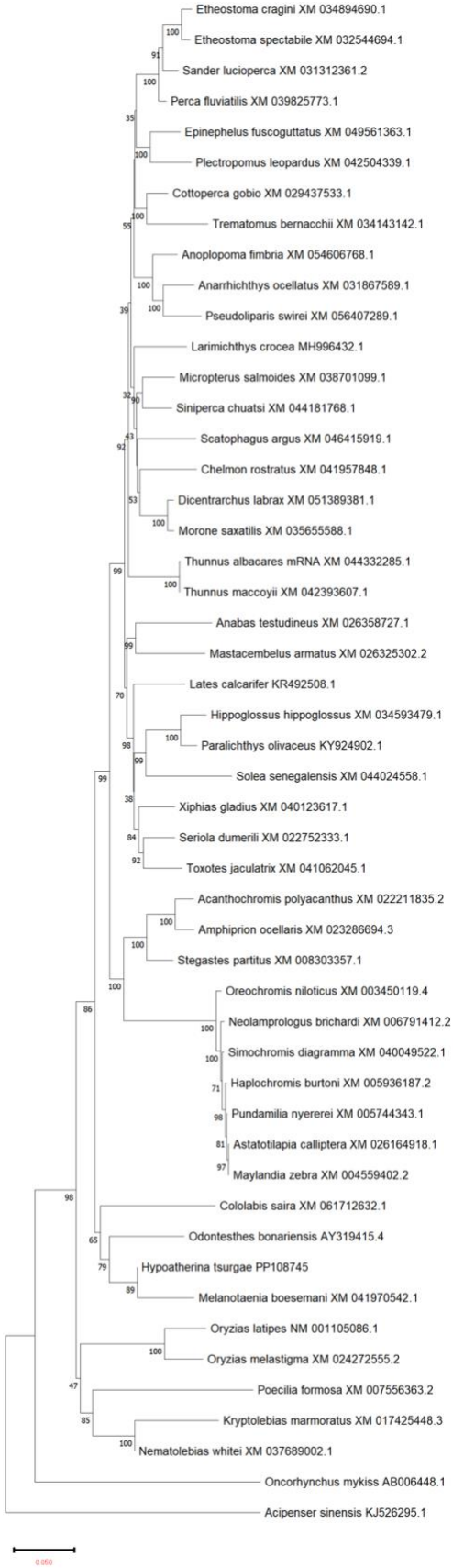


**Fig. 3.** Phylogenetic tree retrieved with the *sox9* mRNA sequence of 50 different fish species along with *H. tsurugae*.

## Gene expression analysis

The result of qRT-PCR illustrated that in *amhy*- (female) individuals the expression was quite high at 2 wah then sharply decreases whereas in *amhy*+ (male) individuals it begins from baseline at 0 wah and gradually increases in an exponential fashion until complete differentiation of gonads occur (Fig. 4).

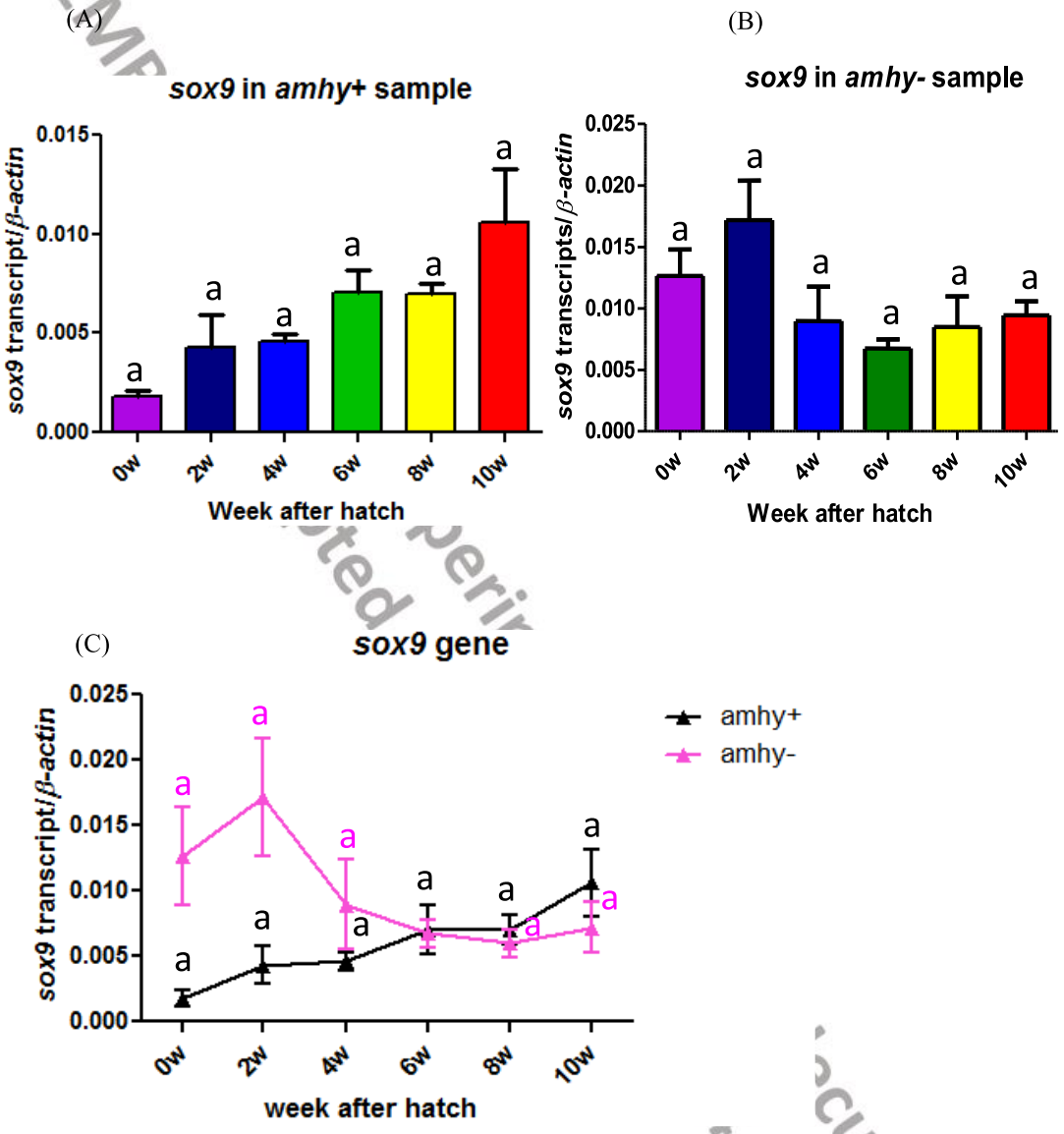


**Fig. 4.** Quantitative mRNA expression of *sox9* gene (a) in *amhy*+ individual (male) (b) *amhy*- individual (female) (c) Both in *amhy*+ and *amhy*- plotted in same graph. Values represent the mean ± SEM of 5-7 fish per time point.

The histological sections of gonads in different larval stages showed that differentiation of gonads male/female is decided at 6 wah. In this stage the primary oocytes are clearly recognized (Fig. 5) which is also correlated with expression of *sox9* gene.

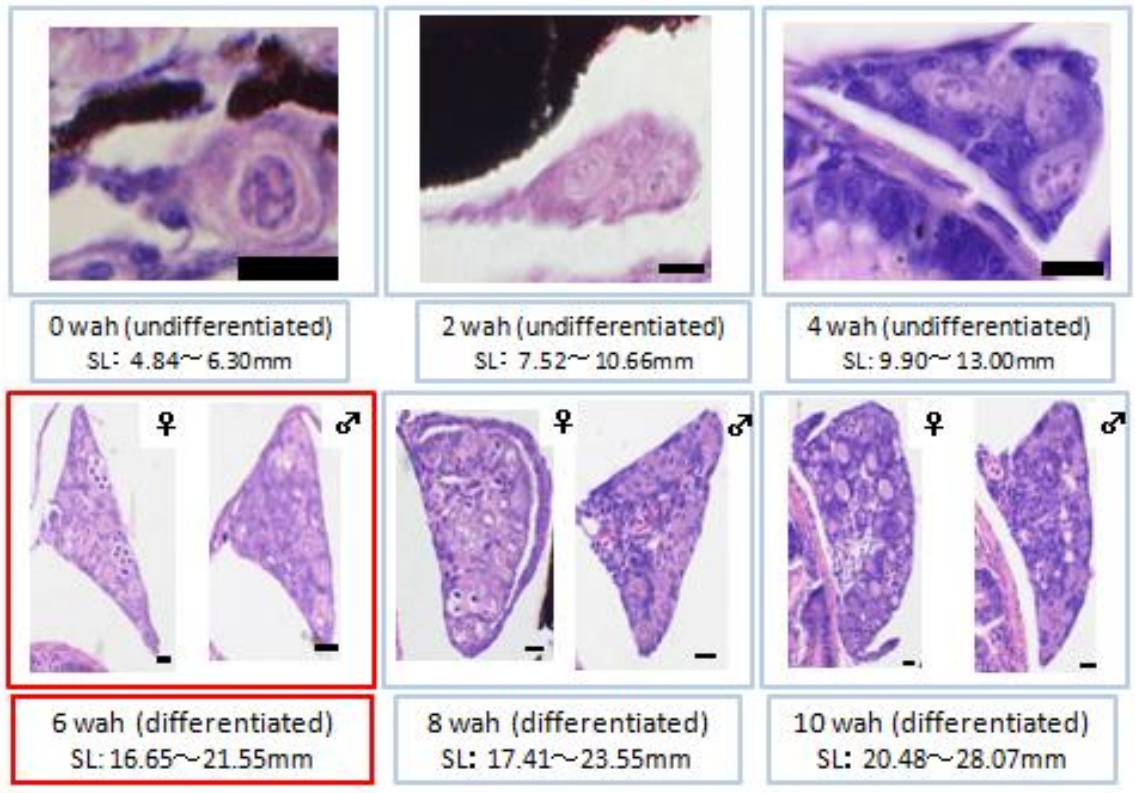


**Fig. 5.** Histological differentiation of gonad *amhy*+ (male) and *amhy*- (female) analyzed every two weeks after hatch.

## Discussion

In the present study, the *sox*9 gene of *H. tsurugae* has been successfully cloned and sequenced. The *sox*9 mRNA is 1454 bp encoding a 479 aa protein. The *sox*9 gene has very close similarity with *sox*9 gene of *Melanotaenia boesemani*, *Stegastes partitus*, *Odontesthes bonariensis*, *Xiphias gladius*, *Seriola dumerili*, *Lates calcarifer*, *Dicentrarchus labrax*, and *Oreochromis niloticus*. The number of *sox*9 subtypes is not the same in different species (Luo *et al*. 2010). There is only one type of *sox*9 gene found in higher vertebrates but usually two subtypes are found in teleosts, likely due to genome duplication (Hu *et al*. 2021, Meyer *et al*. 2005). It is presumed that there may be two types of *sox*9 genes in *H. tsurugae* but only one type was cloned in this study. The phylogenetic analysis revealed that our sequence forms a clade with another Atheriniformes, *Maelanotaenia boesemani*.

In this study, focus is given to the expression pattern of the *sox*9 gene in gonads of *H. tsurugae*. From the qRT-PCR result, the expression of the *sox*9 gene is correlated with the expression of the *amhy* gene that significantly reached a peak at 6 wah, then decreases (Bej *et al*. 2017). The expression of *amhy* was detected from before the appearance of first signs of histological differentiation in presumptive Sertoli cells surrounding germ cells in the undifferentiated gonads. Similarly, the expression of *sox9* in *amhy*+ (male) begins from baseline at 0 wah and is expressed in an increasing fashion needed for the male developmental pathway for testis differentiation. In *amhy*- (female) individuals though highly expressed in the initial stage (0 wah) and the expression reaches a peak at 2 wah it declines afterwards which indicates the low expression needed for differentiation of female gonads, the ovary. It has been reported in the expression profile of the Zebra fish that the *sox9* gene reaches a peak at 18 days post hatch which is significantly different from male sex individuals and after 18 days after hatch it decline abruptly (Jorgensen *et al*. 2008). The expression of *sox9a* and *sox9b* was also studied in medaka

fish and it is expressed in testis and ovary during the developmental period of gonads (Klüver *et al*. 2005). However, the study of the expression of *sox9* gene in the medaka fish gave inconsistent results (Yokoi *et al*. 2002, Nakamoto *et al*. 2005). In the rice field eel, *Monopterus albus*, the *sox9* gene is also expressed in both testis and ovary during the developmental period (Zhou *et al*. 2003). The *sox9* gene is over expressed in testicles as compared to the ovary in *Acipeneser sturio* and *Acipenser fulvescens* (Berbejillo *et al*. 2012, Burcea *et al*. 2018). It has been reported that *sox9* is involved in many signaling pathways during sex determination/differentiation period and in gonad development of embryo and adult.

## Conclusion

From the above study, it may be concluded that expression of *sox9* is essential for development and differentiation of male sex organ testis and it is assumed that after the trigger of sex determining gene *amhy* switches on, the next cascade is performed by *sox9* gene and other sex related genes to differentiate the gonad during sex differentiation period for *H. tsurugae*. However, more functional experiments are necessary to understand the mechanisms of downstream pathways of gene regulation during gonadal differentiation period of this species.

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